CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10)

Applicant:	Ensoli
For:	Novel TAT Complexes, and Vaccines Comprising Them
Filed:	August 11, 2006
Application No.	10/597,926
Confirmation No.	7925
Attorney Docket No.	114-06

I hereby certify that the following documents:

(1) Submission of Certified Copy of Priority Document Cover Letter - 1 page
(2) Certified Copy of Priority Document (11 duplex pages)
(3) Postcard Acknowledgement of Receipt

are being deposited with the United States Postal Service **Express Mail Post Office to Addressee** service under 37 CFR 1.10 in an express mail envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on May 8, 2007.

Laura L. McGee

EV 833 185 675 US
Express Mail Tracking Number

5-9-07 IFW 1648

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/597,926	Confirmation No.: 7925
Applicant	:	Ensoli	
Filed	:	August 11, 2006	
Art Unit	:	1648	
Examiner	:	Not yet assigned	
For	:	Novel TAT Complexes, and Vaccines Comprising Them	
Docket No.	:	114-06	
Customer No.	:	23713	

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage for **Express Mail** in an envelope addressed to: Commissioner for PatentsPO Box 1450, Alexandria, VA 22313-1450

May 8, 2007
Date

Laura L. McGee

EV 833 185 675 US
Express Mail Tracking Number

SUBMISSION OF CERTIFIED COPY OF PRIORITY DOCUMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicant respectfully submits herewith, a certified copy of GB 0405480.5. The present application is filed under 35 U.S.C. 371, based on PCT PCT/EP2005/003043, which application claims priority to GB 0405480.5. A postage-paid return receipt postcard to be stamped by the United States Patent and Trademark office is also enclosed.

It is believed that the present submission does not require the payment of any fees. If this is incorrect, however, please charge any fees required by 37 C.F.R. 1.16-1.17 to Deposit Account No. 07-1969.

Respectfully submitted,

Donna M. Ferber
Reg. No. 33,878

GREENLEE, WINNER AND SULLIVAN, P.C.
4875 Pearl East Circle, Suite 200
Boulder, CO 80301
Telephone: (303) 499-8080
Facsimile: (303) 499-8089
E-mail: winner@greenwin.com
Attorney Docket No. 114-06
May 8, 2007

The Patent Office
For Innovation

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

I further certify that attached hereto is a true copy of the entries made to date in the Register of Patents in respect of the patent.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Andrew Gerry

Dated 9 August 2006

Patents Form 1/77

Patents Act 1977
(Rule 16)

The Patent Office

12MAR04 E880193-1 003312
P01/7700 0.00-0405480.5 ACCOUNT CHA

THE PATENT OFFICE
H
11 MAR 2004
LONDON

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference GBP89320

2. Patent application number
(The Patent Office will fill in this part) 0405480.5 11 MAR 2004

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Istituto Superiore di Sanità,
Viale Regina Elena 299
00161 Roma
Italy

Patents ADP number (if you know it) 8609171900 1

If the applicant is a corporate body, give the country/state of its incorporation Italy

4. Title of the invention NOVEL TAT COMPLEXES, AND VACCINES COMPRISING THEM

5. Name of your agent (if you have one)
"Address for service" in the United Kingdom to which all correspondence should be sent
(including the postcode)

Marks & Clerk
90 Long Acre
LONDON
WC2E 9RA

ADP No. 00000018001

Patents ADP number (if you know it)

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months

Country	Priority application No (if you know it)	Date of filing (day / month / year)

7. Divisionals, etc: Complete this section only if this application is a divisional, application or resulted from an entitlement dispute

Number of earlier application	Date of filing (day / month / year)

8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request? Yes

(Answer 'Yes' if:
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Patents Form 1/77

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	0
Description	14
Claim(s)	2
Abstract	1
Drawing(s)	

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
Request for preliminary examination and search (Patents Form 9/77)	1
Request for substantive examination (Patents Form 10/77)	
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

Signature(s) Marks & Clerk Date: 11 March 2004

12. Name and daytime telephone number of person to contact in the United Kingdom

Patent Chemical Formalities
020 7400 3000

NOVEL TAT COMPLEXES, AND VACCINES COMPRISING THEM

The present invention relates to the use of protein materials comprising the gp120 V3 loop in the manufacture of a vaccine against viruses expressing gp120.

HIV adsorption to the membrane of target cells occurs upon the interaction of HIV gp120 with the cell receptor, CD4. This interaction induces a conformational transition in gp120, leading to the exposure of the gp120 V3 loop. According to one proposed model, the gp120 V3 loop interacts, in turn, with other cell surface molecules acting as co-receptors for HIV. The most important HIV co-receptors are the chemokine receptors CCR5 and CXCR4. It is now generally accepted that macrophage-tropic (M-tropic) HIV isolates infect macrophages *via* CCR5, while T cell line tropic (TCL-tropic) HIV strains infect TCL *via* CXCR4, and dual-tropic strains infect *via* either of the co-receptors.

The interaction of the gp120 V3 loop with the co-receptors for HIV allows the formation of a ternary complex between the co-receptor, CD4 and gp120 leading, in turn, to conformational changes in gp41. These conformational changes are believed to be required for the exposure of the HIV fusion peptide present at the N terminus of gp41, which can interact with the cell surface with ensuing fusion between the virus envelope and the cell membrane. In the course of this stepwise mechanism, cryptic epitopes on gp120 are exposed as a result of the interaction between gp120 and CD4. These epitopes are otherwise hidden, and are recognised by antibodies directed against the portion of gp120 interacting with the co-receptors.

These cryptic epitopes are the object of intense research for vaccination and passive-immunisation purposes, and there is considerable evidence that the gp120 V3 loop is involved in co-receptor recognition and usage. In particular: point mutations or deletions in V3 have been shown to abrogate or shift co-receptor usage; V3 peptides have been proven to interact with CXCR4; and antibodies against V3 can impair or block gp120-CCR5 binding. This model is, therefore, generally accepted, although various observations have lead to the notion that additional events are involved in co-receptor utilisation, particularly in macrophages.

Tat is a regulatory protein associated with human immunodeficiency virus type 1 (HIV-1), produced very early after infection, and which is essential for virus gene expression, replication and infectivity (Arya 1985; Fisher 1986; Chang 1995). During

acute infection of T cells by HIV, Tat is also released in the extracellular milieu and taken up by neighbour cells (Frankel 1988; Ensoli 1990; Ensoli 1993; Chang 1997) where, depending on the concentration, conformational state, and cell type, it can increase virus infectivity. Specifically, uptake of Tat can enhance, in infected cells, virus gene expression and replication (Frankel 1988; Ensoli 1993; Chang 1997), while, in uninfected cells, it enhances the expression of both co-receptors CCR5 and CXCR4, favouring transmission of both macrophage and T lymphocyte-tropic HIV-1 strains (Huang 1998; Secchiero 1999).

Consistent with these findings, the immune response to Tat has been shown to play a key role in controlling the progression of AIDS and AIDS-associated diseases, and to protect Tat-vaccinated monkeys from SHIV infection (Cafaro *et al.*, Nat Med 1999). However, no specific role of Tat in the molecular events mediating HIV adsorption or membrane fusion has been ever recognised or postulated, on the basis of the available studies.

Surprisingly, we have found that Tat can interact with the gp120 V3 loop, mimicking the CCR5 co-receptor, both at the molecular (structural) and functional level, thereby conferring on CCR5-tropic HIV strains the ability to infect even CCR5-negative cell targets.

Thus, in a first aspect, there is provided a complex comprising first and second peptides, the first peptide comprising the V3 loop of gp120, the V3 loop being available to coordinate with a binding region on the second peptide, the binding region being derived from Tat and being recognisable by the monoclonal antibody directed against the CCR5 second extracellular loop described by Lee, B., *et al.*, J Biol Chem, 1999, for use in therapy. The monoclonal antibody identified is available from Pharmingen under catalogue no. 36460D.

It is particularly surprising that Tat is able to bind the V3 loop of gp120 and that, furthermore, the region of Tat that binds the V3 loop is recognisable by anti-CCR5 antibodies. Given the exceedingly high specificity of monoclonal antibodies, it is highly likely that the effect of Tat on the V3 loop of gp120 is similar, or identical, to that of CCR5. Indeed, we have established that extracellular Tat is effectively able to provide

CCR5 functionality to susceptible T cells that do not express this co-receptor, at least insofar as HIV infectivity is concerned.

Thus, M-tropic HIV strains will initially target only macrophages but, once Tat is released, $CCR5^-$ T cells can rapidly be infected, which can lead to the massive build up of virus necessary to establish persistent infection.

By recognising this, it is now possible to provide an antigenic complex of at least the relevant parts of Tat and gp120, thereby to stimulate an immunogenic response in an individual, for prophylaxis or therapy.

Such complexes may also be used to generate antibodies for use in passive immunisation, such as where it is suspected that an individual may have been exposed to HIV. Such antibodies may be raised in animals for use in humans, and may also be sequenced and humanised by methods well known in the art.

While complexes and antibodies against such are of particular use against CCR5-tropic viral strains, they may also be employed against TCL-tropic strains to hamper, or even block, Tat-mediated spread of the virus from one T cell to another. Likewise, dual-tropic strains may also be targeted.

Owing to the molecular mimicry of CCR5 by Tat, complexes and antibodies generated thereagainst will be also in part directed against cryptic epitopes present in CCR5 or in the CCR5/V3 loop complex, contributing further to the efficacy of the vaccine or antibodies used in passive immunisation.

A complex of the present invention may generally be suitably provided as a combination of two peptide species in a vehicle suitable for injection. The vehicle containing the complex may be stored as such, or may be provided as separate preparations of the individual peptides and/or vehicle for combination prior to use.

The complex of the present invention will typically comprise the two peptide species in contact with each other. Whilst it is preferred, it is not necessary that the two species be present in stoichiometric amounts, nor that even a majority of either species be complexed or bound to the other. All that is required is that a sufficient amount of an antigenic combination of the two species be presented in order to be able to stimulate an immune response thereagainst.

The complex of the present invention may rely simply on the natural interaction between Tat and the V3 loop of gp120. Weaker complexes may also be employed, but it is generally preferred to strengthen the complex. In this respect, for example, it is possible to employ the disulphide bridges that can occur in association with the cysteine-reach region of the Tat protein.

The complex may simply comprise the relevant areas of Tat and gp120. In the case of gp120, all of, or a substantial part of, the V3 loop region, is sufficient although, as indicated by molecular docking data, residues proximal to the V3 loop may also be involved, *in vivo*. In the case of Tat, while amino acids 1 to 61 and, possibly, up to amino acid 70 and beyond, are involved in binding with the V3 loop, it appears particularly advantageous to employ residues 21 to 58. This stretch of the Tat sequence binds particularly strongly to anti-CCR5 antibodies.

What is important is that the complex be adequate to stimulate an immune response such that antibodies raised thereby will recognise the Tat/gp120 complex *in vivo*. Thus, while it is generally possible to employ Tat sequence 21 to 58 to bind the gp120 V3 loop, it is possible that antibodies raised against the resulting complex will not recognise the complex of Tat and gp120 *in vivo*.

Thus, it is generally preferred that the complex employed for raising antibodies, or as an immunogen in a vaccine preparation, comprises substantially the full sequence of Tat in an immunologically natural conformation. In this regard, it is possible to make certain amino acid substitutions without affecting the immunogenicity of Tat, although such substitutions may affect the biological efficacy of the resulting Tat in other ways. It may be desirable to make such substitutions for reasons such as ensuring greater binding between Tat and the V3 loop, for example, or such substitutions may result from preferred genetic engineering processes.

It is less important that the V3 loop be part of the overall gp120 molecule, and this loop may be provided in a suitable context in a carrier molecule, provided that it is available in such a fashion as to be able to form a complex with the Tat peptide. In particular, it will be appreciated that such a carrier molecule may express more than one V3 loop to form a multimeric complex with various Tat proteins.

In general, it is preferred that naturally occurring gp120, or a similar or related protein, such as a variant or engineered mutant thereof, be employed, provided that, conformationally, it exposes the V3 loop. This may be achieved by adding CD4, or the gp120 binding epitope of CD4, to a preparation of the other two peptide species, thus enabling gp120 to expose the V3 loop in such a system.

In a preferred aspect, the present invention provides use of a complex as described above in the preparation of a medicament for the treatment or prophylaxis of a viral infection, whereby the infecting virus expresses a molecule capable of forming a ternary complex between itself, CD4 and CCR5.

In general, the target virus for this treatment or prophylaxis will be a strain of HIV or HTLV, but also may be an animal strain, such as SHIV, for example.

Antibodies against complexes of the present invention may be raised by standard means, and suitable monoclonal or polyclonal antibodies, preferably monoclonal, generated. It is preferred that such antibodies are capable of binding none of CCR5, Tat, gp120, or the V3 loop region of gp120, individually, but are capable of binding a complex of Tat and gp120. Thus, the resulting antibodies can bind and block complexes of Tat and gp120 *in vivo*, thereby preventing infection. It will be appreciated that such antibodies may not necessarily bind both, or all, of the components of the complex, and may simply bind cryptic epitopes exposed on binding of Tat with gp120. Such epitopes may occur on Tat, gp120 or even CCR5, and this represents an advantage of the invention.

In the preparation of suitable antibodies against the complexes of the invention, antibodies that bind epitopes normally present on either Tat or gp120 can be removed by the simple expedient of eliminating antibodies or lines that bind Tat, gp120, or the V3 loop of gp120, individually, from the polyclonal preparation or those monoclonal lines selected, thereby leaving only polyclonal preparations, or lines expressing antibodies, that bind epitopes present only in the complex.

It will be appreciated that monoclonal antibodies raised in this manner, if raised in animals, may be suitably humanised by methods well known in the art. The present invention extends to polyclonal, monoclonal and humanised antibodies specific for the complexes described herein.

Active vaccines comprising complexes of the invention are provided, as are antibody preparations for passive immunisation comprising antibodies of the invention, and vehicles suitable for such vaccines are well known in the art, and may comprise suitable substances, such as stabilisers, isotonic agents, antibacterial agents.

The present invention also extends to the complexes themselves.

Thus, the Tat-V3 loop complex provides a novel antigen that can be used for preventive or therapeutic vaccination by inducing protective antibodies capable of blocking the *in vivo* Tat-V3 loop interaction. Antibodies may also block the CCR5-V3 loop interaction, and the complex may be used to generate protective antibodies for passive immunisation to block mother-to-child transmission or horizontal HIV transmission in exposed individuals, for example.

In a preferred embodiment, the present invention provides a molecular complex formed between the HIV Tat protein and the HIV envelope protein gp120, which is generated upon the interaction of the cysteine rich and basic regions of Tat and the gp120 V3 loop. Molecular complexes obtained using the whole Tat protein, its mutants, fragments or derivatives thereof, and the HIV envelope protein gp120, fragments or derivatives thereof, are preferred, as are their use as antigens for preventive or therapeutic vaccination against HIV/AIDS.

A preferred complex comprises HIV Tat cysteine and basic region and the V3 loop of HIV Env. Another comprises HIV Tat fragments or derivatives thereof and HIV Env fragments or derivatives thereof, while another comprises HIV Tat peptides or epitopes and HIV Env peptides or epitopes.

Also preferred is a covalently linked chimera between HIV1 Tat, fragments or derivatives thereof, and HIV Env, fragments or derivatives thereof.

The Tat component, in one embodiment, may be a transactivation mutant, for example the Tat-cys_{22} mutant.

The HIV of these embodiments is preferably HIV-1. Preferred clades are HIV-1 clades A, B, C, D, E, F, G, and O. It will be appreciated that the invention extends to CCR5-tropic, CXCR4 tropic and dual-tropic strains, and that reference to any specific virus, or type or class thereof, is equally applicable to all viruses subject of the invention.

The vaccines of the invention are of particular use in prevent or inhibition of HIV transmission from mother to child or between HIV-exposed individuals.

The present invention is further illustrated by the following, non-limiting Examples.

EXAMPLE 1

Molecular Interaction of HIV-1 Tat with the gp120 V3 loop

The binding of HIV-1 Tat to the HIV-1 gp120 V3 loop was investigated using a molecular docking model, in which Tat (BH10 HIV strain) was allowed to interact with the V3 loop of the Env protein (Ba-L HIV strain). All structural models were calculated using, as template, all of the available structures of the Tat protein and of the Env protein deposited in the Protein Data Bank (Berman, H.M *et al.*, *Nucl. Acids Res. 28*, 235-242, 2000) as of July 2003. The sequences of the various proteins were aligned using ClustalW (Thompson, J.D *et al.*, *Nucl. Acids Res. 22*, 4673-4680, 1994) and the structural models were generated with Modeller6v2 (Sali, A. and Blundell, T.L. *J. Mol. Biol. 234*, 779-815, 1993). All of the calculated structural models were optimised through energy minimisation with AMBER-5 (Pearlman, D.A. *et al.*, in *AMBER 5.0*, University of California, San Francisco, 1997). These structural models were then used to calculate the protein-protein adducts with the program BIGGER (Palma, P.N. *et al.*, *Proteins Struct. Funct. Genet. 39*, 372-384, 2000). The latter program generates protein-protein complexes and ranks them on the basis of shape complementary and non-bonded (electrostatic and Van der Waals) interactions.

Initial molecular docking calculations were made with the isolated V3 loop (Aa 100-139) and gave rise to three types of low energy adducts, characterised by three unique interaction regions. These adducts are characterised by different Tat residues interacting with the V3 loop although, by contrast, the V3 loop showed only a single interaction region involving residues Thr103, Arg104, Ala134, His135, Asn137, and several amino acids of the 119-131 segment of the V3 loop.

The interaction between Tat and a relatively large domain of gp120 exposing the V3 loop (Aa 167-458) was next calculated. As no structural information on the conformation of the V3 loop and its relative orientation with respect to the rest of the gp120 domain was available, the range of accessible conformations and the flexibility for V3 loop was sampled. The variability of the loop conformation can, in fact, have a sizable effect on the complex geometry. The conformation sampling was done through a long molecular dynamics simulation in explicit solvent. Docking calculations were performed, allowing Tat to interact with five different conformations of the gp120 of the Env protein including the two most different V3 loop conformations plus three intermediate conformations.

These calculations identified an adduct interacting with Tat in a region involving the V3 loop, which was essentially the same as that found in one of the adducts found with calculations performed with the V3 loop alone. This adduct is, therefore, predicted to be the most stable and was subjected to molecular dynamics (MD) calculations to optimise its conformation and to estimate its stability when a complete force field (produced by the atoms of the two molecules) is effective. The calculations were performed on both the oxidised (*i.e.* with disulphide bridge on V3 loop) and the reduced states of the Env protein and showed that the adduct is similarly stable in both oxidation states. In order to validate the interaction model found with the described procedure, and to analyse the protein-protein interface, docking calculations with the program Haddock were also performed. The five lowest energy adducts were found to have the same geometry as the model found with BIGGER calculations.

All these calculations, therefore, pointed at a unique mode of interaction. The final structural model of the adduct was found to be quite stable, with an average interaction surface of 2260 ± 112 $Å^2$ and an average protein-protein intermolecular energy of –412±14 kcal mol^{-1}. The largest contribution to the interaction energy was due to the electrostatic contribution, with an average energy of -325±12 kcal mol^{-1}: the interaction surface involves residues 1- 3, 16, 19- 22, 25, 26, 29, 34, 35, 45- 47, 51, 55, 57, 59, 61 on Tat and residues 104, 119, 120, 121, 124, 125, 127, 128, 130-135, 208, 210, 215, 219, 220-222 on the V3 loop.

Three intermolecular salt bridges between Tat and gp120 residues, respectively (Asp5-Arg119, Lys50-Glu210 and Lys19-Asp130) were found to be completely conserved in all of the various models and during the molecular dynamics simulations. The adduct

was found to be further stabilised by additional intermolecular hydrogen bonds, which varied in number during the simulation from six to eleven, but always involving at least one residue of the common interaction region. A sizable contribution to the stabilisation of the adduct was also determined to be brought by 30 to 40 hydrophobic interactions. Twenty to thirty of these interactions were contributed by residues belonging to the V3 loop whereas about 20 of them by residues belonging to the 20-59 segment of Tat.

EXAMPLE 2

Tat binds the gp120 V3 loop in an ELISA assay.

Enzyme-linked immunosorbent assay (ELISA) tests were performed to determine whether Tat actually binds the gp120 V3 loop *in vitro*. To this purpose, ELISA plates were coated with a peptide encompassing the entire V3 loop, followed by extensive blocking with carrier bovine serum albumin (BSA), multiple washing steps, and additional incubation with biologically active Tat protein or, as a control, its buffer (PBS-BSA 0.1%) (Cafaro *et al.*, Nat Med 1999; Fanales-Belasio *et al.*, Immunology 2001). Monoclonal anti-V3 and polyclonal anti-Tat antibodies were used as primary antibodies for the detection of the bound protein.

When uncoated (*i.e.* BSA-blocked) wells were used in the ELISA assay with anti-Tat or anti-V3 antibodies, a slight background signal was detected, ranging from about 0.1 to 0.4 OD. The results are shown in Table 1, below. However, when wells coated with the V3 peptide were incubated with Tat, the signal was increased to about 1 optical density (OD). In contrast, wells incubated with buffer alone yielded signals comparable to background levels (uncoated wells). As expected, V3 coated wells yielded high ELISA signals with anti-V3 antibodies. These experiments show that biologically active Tat binds to the gp120 V3 loop *in vitro*, confirming the data obtained with molecular docking calculations.

TABLE 1			
		Antibodies	
Coating	Incubation	anti-Tat	anti-V3
none	buffer	0.132 OD	0.15 OD
none	Tat	0.431 OD	0.122 OD
V3 (500 ng)	buffer	0.277 OD	3 OD
V3 (500 ng)	Tat	1 OD	3 OD

EXAMPLE 3

Tat is recognised by antibodies directed against the CCR5 HIV co-receptor

Since the gp120 V3 loop appears to be the major determinant for co-receptor choice and utilisation by HIV strains, experiments were performed to determine whether the capability of Tat to bind the V3 peptide was due to mimicry by Tat of co-receptor molecules. To this purpose, monoclonal antibodies directed against the major HIV-1 co-receptors (CCR5 and CXCR4) (Pharmingen) were used in an ELISA assay to determine whether they could recognise Tat, or Tat peptides consisting of specific Tat sequences and/or structural and functional domains. These monoclonal antibodies are known to recognise conformational epitopes present on HIV-1 co-receptors (Lee B *et al.*, J Biol. Chem., 1999; Baribaud F *et al.*, J Virol. 2001). Accordingly, any recognition of Tat by these antibodies would indicate that Tat shares structural similarity with the relevant co-receptor.

ELISA plates were coated either with native Tat or one of the following Tat peptides:

Tat (1-20) (N-terminal domain);

Tat (21-40) (cysteine-rich region - transactivation domain);

Tat (36-50) (core region);

Tat (46-60) (basic region - nuclear localisation signal);

Tat (56-70) (glutamine-rich region);

Tat (65-80) (RGD sequence);

Tat (73-86) (RGD sequence);

Tat (83-102) (C-terminal domain); and

Tat (21-58) (cysteine, core, and basic regions).

Monoclonal anti-CCR5 or anti CXCR4 antibodies were used for the detection step. Anti-CCR5 specifically recognised the recombinant native Tat protein, the Tat (21-58) peptide and, although with a lower efficiency, the Tat (46-60) peptide. The results are shown in Table 2, below. In contrast, no recognition was observed with the antibodies directed against CXCR4.

TABLE 2

Coating	Antibodies			
	anti-CCR5	anti-CXCR4	CTR isot.	anti-Tat
Tat	1.452	0.085	0.081	3.000
Tat 1-20	0.000	0.000	0.006	3.000
Tat 21-40	0.000	0.133	0.082	0.1
Tat 36-50	0.000	0.000	0.000	0.000
Tat 46-60	0.466	0.000	0.063	0.000
Tat 56-70	0.147	0.000	0.000	0.75
Tat 65-80	0.000	0.000	0.000	0.1
Tat 73-86	0.000	0.077	0.019	0.087
Tat 83-102	0.000	0.058	0.000	0.000
Tat 21-58	3.000	0.072	0.108	0.551

The anti-CCR5 antibody used in these experiments is known to recognise a conformational epitope present in the CCR5 second extracellular loop (ECL2) and to be neutralising for HIV (Lee B *et al.*, J Biol. Chem., 1999). In addition, RCL2 is known to be involved in the gp120 conformational changes leading to membrane fusion (Lee B *et al.*, J Biol. Chem., 1999). Thus, these data indicated that Tat sequences encompassing both Tat transactivation domain and basic-rich region mimic at the structural level a region of the CCR5 involved in cell fusion upon recognition of CCR5 by gp120.

EXAMPLE 4

Native, biologically active Tat is required for CCR5 recognition by anti-CCR5 antibodies

The data described in Example 3, above, indicate that Tat sequence present in peptide 21-58 is capable of folding to mimic a conformational epitope of the CCR5 co-receptor. To determine whether a specific conformation of Tat is required for recognition by the anti-CCR5 antibody, the capability of native, biologically active Tat to be recognised by the anti-CCR5 antibody was compared to an oxidised Tat preparation, obtained by exposing the protein to the air and direct light, according to a procedure known to abrogate most of its biological activity (Fanales-Belasio, Immunology, 2001). This procedure results in the oxidation of -SH groups and in the formation of intra- and inter-molecular disulphide bounds, mediated by the cysteine residues present in the Tat transactivation domain. The transactivating properties of Tat, in turn, are known to activate the expression of host genes including HIV co-receptors (Huang 1998; Secchiero 1999). However, Tat transactivation properties are abolished in a transactivation mutant where cysteine 22 is substituted by a glycine (*Tat-cys*$_{22}$) (Caputo A *et al.*, Gene Ther. 1996). This Tat mutant, nevertheless, maintains its immunogenic properties, intact (Caselli E *et al.*, J Immunol. 1999). Thus, *Tat-cys*$_{22}$ was also included in this set of experiments.

ELISA wells were coated with native Tat, oxidised Tat (Tat OX) or *Tat-cys*$_{22}$ and the anti-CCR5, anti-CXCR4 antibody were used in the detection step. These experiments showed that the antibody specifically recognises the recombinant native Tat protein and the *Tat-cys*$_{22}$ mutant, but not the oxidised Tat protein. The results are shown in Table 3, below. In contrast, and as a control, polyclonal (rabbit) anti-Tat antibodies recognised, as expected, all proteins with similar efficiency, demonstrating that all wells were equally coated.

TABLE 3

Coating	Antibodies			
	anti-CCR5	anti-CXCR4	CTR isot.	anti-Tat
Tat	1.24	0.042	0.051	3
Tat OX	0.128	0.071	0.016	3
Tat-cys22	0.75	0	0.026	3

These experiments showed, therefore, that Tat sequences encompassing the Tat transactivation domain, the core region and the basic region, fold to mimic a major epitope present on CCR5, and that a point mutation which abrogates the transactivating properties of Tat does not interfere with epitope formation.

EXAMPLE 5

Extracellular Tat enhances infection of CD4+ susceptible cells by HIV-1 and expands HIV-1 tropism in CCR5 negative cell lines.

To determine whether Tat can mediate HIV-1 entry by mimicking CCR5, it was necessary to determine the effects of Tat on HIV entry in a CCR5-independent system. To this purpose, infection experiments were performed with a single cycle assay using a replication-defective recombinant HIV-1 encoding a cloramphenicol acetyltransferase (CAT) reporter gene and which was pseudotyped with the envelope glycoprotein of the CXCR4-tropic HXBc2 HIV isolate, or the CCR5-tropic ADA or YU2 HIV isolates. These replication-defective viruses (herein referred to as the R4-tropic HXBc2/HIV-CAT or the R5-tropic ADA/ or YU2/HIV-CAT viruses) enter susceptible cells through CD4/CXCR4 or CD4/CCR5, integrate their cDNA's in the cell genome, and express the reporter gene CAT, but they can not produce progeny, *i.e.* they cannot support further infection of cells through subsequent cycles of virus production (Helseth E. J Virol 1990)). Thus, HIV-CAT viruses produce a single-round infection cycle of target cells, quantification of CAT acetylation levels allowing quantitative evaluation of the efficiency of HIV infection .

Based on the data obtained in Examples 1 to 4, above, experiments were performed to determine whether Tat could assist infection by HIV, expand HIV tropism, and render TCLs susceptible to infection by R5-tropic (*i.e.* monocyte/macrophage-tropic) HIV strains, owing to molecular mimicry of CCR5 co-receptor by Tat. To this purpose, CEMss and Jurkat cells, two TCLs expressing both CD4 and CXCR4, but lacking CCR5 expression, or the CD4-negative 293 cell line, were plated on Tat-coated wells that had previously been incubated with HIV-CAT viruses pseudotyped with the envelope from the X4-tropic HXBc2 strain, or the CCR5-tropic ADA or YU2 strains. As expected, both CEMss and

Jurkat cell lines were efficiently infected with the HXBc2/HIV-CAT, whereas no infection was detected with the CD4-negative 293 cells, due to the lack of the primary HIV-1 receptor. Strikingly, furthermore, both CEMss and Jurkat cells were also infected at high efficiency by the ADA or YU2 pseudotyped HIV-CAT, despite being known to be resistant to infection by R5-tropic HIV-1 strains. These data, therefore, confirmed the unexpected prediction that immobilised Tat is capable of increasing HIV-1 cell tropism through molecular mimicry of specific CCR5 extracellular structural domains, *i.e.*, of rendering CCR5-tropic strains capable of infecting CCR5-negative TCLs.

EXAMPLE 6

Anti-CCR5 antibodies, but not anti CXCR4 antibodies, block Tat-assisted infection of CCR5-negative cell lines

To further demonstrate that immobilised Tat expands the cell tropism of R5-tropic HIV-1 strains by mimicking CCR5, experiments were performed to determine whether active molecules capable of blocking CCR5 were also capable of blocking Tat-assisted infection of CCR5-negative cells. To this purpose, the CD4+/CCR5- CEMss cells were plated in the presence of antibodies directed against CCR5, CXCR4 or CCR3 on Tat-coated wells which were previously incubated with cell supernatants containing the R5-tropic ADA/HIV-CAT single infection round recombinant virus. Tat assisted infection was almost completely abolished by anti-CCR5 antibody, whereas no reduction in infectivity was observed with anti-CXCR4 or CCR3 antibody as compared to control. Since CEMss cells are CCR5-negative, these data indicate that the blocking activity of the antibodies is due to their capability of recognising Tat structural motives mimicking CCR5 conformational epitopes, as detailed in Examples 3 and 4. Further, these data confirmed that molecular mimicry of CCR5 by Tat is required for entry of CCR5-tropyc HIV-1 strains in CCR5-negative cells.

Claims:

1. A complex comprising first and second peptides, the first peptide comprising the V3 loop of gp120, the V3 loop being available to coordinate with a binding region on the second peptide, the binding region being derived from Tat and being recognisable by an anti-CCR5 antibody,

for use in therapy, particularly for use as an immunogenic component in a vaccine.

2. A complex according to claim 1, wherein the second peptide comprises the HIV Tat cysteine and basic region and the first peptide comprises the V3 loop of HIV Env.

3. A complex according to claim 1, wherein the second peptide comprises HIV Tat fragments or derivatives thereof and the first peptide comprises HIV Env fragments or derivatives thereof.

4. A complex according to claim 1, wherein the second peptide comprises HIV Tat peptides or epitopes and the first peptide comprises HIV Env peptides or epitopes.

5. A complex according to any preceding claim, wherein the complex comprises at least one covalent linkage between the peptides.

6. A complex according to claim 5, comprising a covalently linked chimera between HIV1 Tat, fragments or derivatives thereof, and HIV Env, fragments or derivatives thereof.

7. A complex according to any preceding claim, wherein the binding region on the second peptide comprises amino acid residues 21-58 of Tat, or an immunological equivalent thereof.

8. A complex according to claim 7, wherein the binding region on the second peptide comprises amino acid residues 1-61 of Tat, or an immunological equivalent thereof.

9. A complex according to any preceding claim, wherein the Tat component is a transactivation mutant.

10. A complex according to claim 9, wherein the Tat component comprises the Tat-cys_{22} mutant.

11. Use of a complex according to any preceding claim, in the manufacture of a medicament for the treatment or prophylaxis of a viral infection, whereby the infecting

virus expresses a molecule capable of forming a ternary complex between itself, CD4 and CCR5.

12. Use according to claim 11, wherein the virus is HIV or HTLV.

13. Use according to claim 12, wherein the virus is HIV-1 or HIV-2.

14. Use according to claim 13, wherein the virus is HIV-1 clade A, B, C, D, E, F, G, or O.

15. Use of a complex as defined in any of claims 1 to 10 to produce an antibody thereagainst, and wherein said antibody recognises none of Tat, gp120, or CCR5, individually, but is capable of recognising an epitope present on a complex of Tat and the V3 loop of gp120 when the gp120 is bound to the CD4 receptor.

16. Antibodies produced in accordance with the method of claim 15.

17. Monoclonal antibodies according to claim 16.

18. Humanised monoclonal antibodies according to claim 17.

19. A vaccine comprising a complex according to any of claims 1 to 10.

20. A vaccine comprising an antibody according to any of claims 16 to 18.

21. A complex according to any of claims 1 to 10, substantially free of cells and cellular detritus.

22. A method for the prevention or inhibition of HIV transmission from mother to child or between HIV-exposed individuals, comprising administering a vaccine according to any of claims 19 to 21 to the mother or individual.

ABSTRACT

NOVEL TAT COMPLEXES, AND VACCINES COMPRISING THEM

Complexes comprising HIV Tat and the V3 loop from gp120 Env are immunogenic to prevent or inhibit infection by HIV.